PB83-193565

# Mutagenic and Carcinogenic Potency of Extracts of Diesel and Related Environmental Emissions: Two-Stage Carcinogenesis in Skin Tumor Sensitive Mice (Sencar)

(U.S.) Health Effects Research Lab.
Research Triangle Park, NC

Prepared for

Department of Energy
Washington, DC

1982

U.S. Department of Commerce
National Technical Information Service
NTIS

TECHNICAL REPORT DATA
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| 1. REPORT NO. | 2. | 3. RI |
|---|---|---|
| EPA-600/J-81-620 | JOURNAL ARTICLE | PB83-193565 |

| 4. TITLE AND SUBTITLE | 5. REPORT DATE |
|---|---|
| Mutagenic and Carcinogenic Potency of Extracts of Diesel and related Environmental Enissions: Two-Stage Carcinogenesis in Skin Tumor Sensitive Mice (Sencar) - | 1981 |
| | 6. PERFORMING ORGANIZATION CODE |
| 7. AUTHOR(S) | 8. PERFORMING ORGANIZATION REPORT NO. |
| T.J. Slaga and L.L. Triplett, Stephen Nesnow | |
| 9. PERFORMING ORGANIZATION NAME AND ADDRESS | 10. PROGRAM ELEMENT NO. |
| Health Effects Research Laboratory<br>US Environmental Protection Agency<br>Research Triangle Park, NC 27711 | A9GA1A |
| | 11. CONTRACT/GRANT NO. |
| 12. SPONSORING AGENCY NAME AND ADDRESS | 13. TYPE OF REPORT AND PERIOD COVERED |
| Office of Research and Development<br>Health Effects Research Laboratory<br>US Environmental Protection Agency<br>Research Triangle Park, NC 27711 | 14. SPONSORING AGENCY CODE<br>EPA-600/11 |

15. SUPPLEMENTARY NOTES
Published In: Environmental International, Vol.5, 417-423, 1981

16. ABSTRACT
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17. KEY WORDS AND DOCUMENT ANALYSIS

| a. DESCRIPTORS | b. IDENTIFIERS/OPEN ENDED TERMS | c. COSATI Field/Group |
|---|---|---|
| | | |

| 18. DISTRIBUTION STATEMENT | 19. SECURITY CLASS (This Report) | 21. NO. OF PAGES |
|---|---|---|
| Release to Public | Unclassified | 8 |
| | 20. SECURITY CLASS (This page) | 22. PRICE |
| | Unclassified | |

REPRODUCED BY
NATIONAL TECHNICAL INFORMATION SERVICE
U.S. DEPARTMENT OF COMMERCE
SPRINGFIELD, VA. 22161

*Environment International*, Vol. 5, pp. 417–423, 1981
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0160-4120/81/040417-07$02.00/0
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EPA-600/J-81-620
JOURNAL ARTICLE

# MUTAGENIC AND CARCINOGENIC POTENCY OF EXTRACTS OF DIESEL AND RELATED ENVIRONMENTAL EMISSIONS: TWO-STAGE CARCINOGENESIS IN SKIN TUMOR SENSITIVE MICE (SENCAR)

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Skin tumors can be induced by the sequential application of a subthreshold dose of a carcinogen (initiation phase), followed by repetitive treatment with a noncarcinogenic tumor promoter. There is a very good dose–response relationship between the induction of the number of papillomas per mouse at early times (10 to 20 weeks) by either tumor initiators and promoters and the final carcinoma incidence after a longer latency (20 to 50 weeks) in SENCAR mice. This system not only can be used to determine the tumor initiating and promoting activities of a compound but if the agent is given repeatedly by itself one can also determine if it is a complete carcinogen, i.e., if it has both tumor initiating and promoting activity. In addition, if the agent is given concurrently with a known complete carcinogen or a tumor initiator one can also determine if the agent has cocarcinogenic or cotumor initiating activity or even possibly anticarcinogenic activity. Likewise, if the agent is given concurrently with a known tumor promoter one can determine if the agent has copromoting or antipromoting activity. Using the SENCAR skin carcinogenesis system we have undertaken the determination of the skin carcinogenic, cocarcinogenic, tumor initiating and promoting activities of various diesel emission particle extracts as well as for comparative purposes, standards such as benzo(*a*)pyrene and 12-0-tetradecanoylphorbol-13-acetate and extracts of emissions from a gasoline engine, roofing tar, coke oven and cigarette smoke condensate. Most of the studies are still in progress but some preliminary data is available on the comparative tumor initiating activities of the various samples at 14 weeks. Caterpillar diesel and cigarette smoke condensate were essentially without activity, whereas the Mustang gasoline catalyst and Olds diesel gave extremely low values although they were slightly above background. Roofing tar, coke oven and Nissan Diesel all gave moderate activity at high doses (1 to 10 mg) but when compared to benzo(*a*)pyrene were relatively low values.

## Introduction

Based on knowledge derived from epidemiological studies, it is currently thought that the majority of cancers in humans are caused by environmental factors. Studies with experimental animals along with the epidemiological data have provided evidence that some chemicals in our environment are responsible for a significant proportion of such cancers.

Current information suggest that chemical carcinogenesis is a multistep process with one of the best studied models in this regard being the two-stage carcinogenesis system using mouse skin. Table 1 summarizes some aspects of carcinogenesis in experimental animals. Skin tumors can be induced by the sequential application of a subthreshold dose of a carcinogen (initiation phase) followed by the repetitive treatment with a noncarcinogenic tumor promoter. The initiation phase

Table 1. Carcinogenesis in man and experimental animals.

1. Complete Carcinogenesis
2. Cocarcinogenesis
3. Tumor Initiation
4. Tumor Promotion
5. Additive and Synergistic Effects of Carcinogens, Tumor Initiators and Tumor Promoters
6. Coinitiating and Copromoting Agents
7. Anticarcinogenesis
8. Antiinitiating and Antipromoting Agents

requires only a single application of either a direct or indirect carcinogen at a subthreshold dose and is essentially irreversible, while the promotion phase is brought about by repetitive treatments after initiation and is initially reversible, later becoming irreversible. This system not only can be used to determine the tumor initiating and promoting activities of a compound but if the agent is given repeatedly by itself one can also determine if it is a complete carcinogen, i.e., if it has both tumor initiating and promoting activity. In addition, if the agent is given concurrently with a known complete carcinogen or a tumor initiator one can also determine if the agent has cocarcinogenic or cotumor initiating activity or even possibly anticarcinogenic activity. Likewise, if the agent is given concurrently with a known tumor promotor one can determine if the agent has copromoting or antipromoting activity. Furthermore, like most carcinogenesis systems, skin carcinogens may have additive or synergistic effects. This system has provided an important model for studying carcinogenesis and for bioassaying carcinogenic agents.

Recently, the generality of the two-stage system or multistage carcinogenesis has been shown to exist in a number of systems besides the skin such as the liver, bladder, colon, esophagus, stomach, mammary, displacental as well as cells in culture (Slaga *et al.*, 1978c). The various two-stage systems known and the initiating and promoting agents involved in each system are shown in Table 2. As is apparent, quite a diversity of initiating and promoting agents exists among the various two-stage systems.

*Tumor initiation*

As stated earlier, the tumor initiation phase appears to be an irreversible step which probably involves a somatic cell mutation as evidenced by a good correlation between the carcinogenicity of many chemical carcinogens and their mutagenic activities (Ames *et al.*, 1975; McCann and Ames, 1976). Most tumor initiating agents either generate or are metabolically converted to electrophilic reactants, which bind covalently to cellular DNA and other macromolecules (Miller and Miller

Table 2. Two-stage carcinogenesis systems.[a]

| Organ System | | |
|---|---|---|
| Mouse skin | Polycyclic aromatic hydrocarbons, urethane, direct-acting electrophiles [(epoxides, β-propiolactone, Bis chloromethylether, N-acetyoxyacetylaminofluorene and N-methyl-N′-nitro-N-nitrosoguanidine (MNNG)] | Croton oil, phorbol esters, fatty acids and fatty acid esters, surface-active agents, linear long chain alkanes, tobacco smoke condensate and extracts of unburned tobacco, certain euphorbia macrocyclic diterpenes, citrus oil, anthralin and other phenols |
| Rat & mouse liver | 2-acetamidofluorene, diethylnitrosamine, 2-methyl-N, N′-dimethyl-4-aminoazobenzene | Phenobarbital (DDT); butylated hydroxytoluene (BHT); polychlorobiphenyls (PCB) |
| Mouse lung | Urethan, polycyclic aromatic hydrocarbons | BHT, phorbol |
| Rat Colon | Dimethylhydrazine | Bile acids, high fat diet, high cholesterol diet |
| Rat bladder | N-methyl-N-nitrosourea | Saccharin, cyclamate |
| Rat & mouse mammary gland | Polycyclic aromatic hydrocarbons | Hormones, high fat diet phorbol |
| Rat stomach | N-methyl-N′-nitro-N-nitrosoguanidine | Surfactants |
| Rat esophagus | Diethylnitrosamine | Diet |
| Mouse cell culture systems | Polycyclic aromatic hydrocarbons, radiation | Phorbol esters, saccharin |
| Rat cell culture system | N-methyl-N′-nitro-N-nitrosoguanidine | Phorbol esters |

[a]See Slaga *et al.* (1978c) for details.

1976; Miller, 1978). Previous studies have demonstrated a good correlation between the carcinogenicity of several polycyclic hydrocarbons and their ability to bind covalently to DNA (Brooke and Lawley, 1964; Slaga *et al.*, (1977). The Millers have proposed a significant general theory to explain the initial event in chemical carcinogenesis which states that all chemical carcinogens that are not electrophilic reactants must be converted metabolically into chemically reactive electrophilic form which then reacts with some critical macromolecule to initiate the carcinogenic process (Miller and Miller, 1976; Miller, 1978).

*Cocarcinogenesis and tumor promotion*

The term cocarcinogenic action means the augmentation of carcinogenesis by a noncarcinogenic agent applied concomitantly with a carcinogen (Van Duuren *et al.*, 1978). A promoting agent is one applied repeatedly after a single dose of a tumor initiating agent that results in tumors (Van Duuren *et al.*, 1978). Tumor promoters can be either weak carcinogens or noncarcinogens. Some cocarcinogens such as croton oil, phorbol esters and anthralin are also tumor promoters. A cocarcinogen primarily has an influence on the initiation of the carcinogenic response through an effect on the carcinogen (permeability, metabolism or repairability of the carcinogenic damage) or the target tissue (for example, hormonal stimulation of mammary gland growth and croton oil stimulation of epidermal growth). A wide variety of agents have been found to be cocarcinogenic in experimental animals such as aliphatic hydrocarbons, aromatic hydrocarbons, phenols, long-chain acids, alcohols, hormones, radiation, viruses, food additives and diet, itself (Slaga *et al.*, 1978c). Table 3 lists some of the more important cocarcinogenic agents that have been tested (Slaga *et al.*, 1978c; Van Duuren *et al.*, 1978). Due primarily to the ease of determining cocarcinogens as well as promoters in the skin system, most of the cocarcinogens have been discovered using that system.

Refer to Table 2 which summarizes the various two-stage systems used and the various promoting agents associated with them. In many cases it is very difficult to distinguish between a cocarcinogen or a promoter because many of the agents have not been critically tested.

The phorbol ester tumor promoters are the most potent of the known tumor promoters, having been shown to have many cellular and biochemical effects (Slaga *et al.*, 1978c; Boutwell, 1964; Boutwell, 1974). Some of the most important effects which seem to be intimately associated with the phorbol ester tumor promoters' action are as follows: (a) they induce changes in the phenotype of normal cells that mimic features of transformed cells; (b) they induce dedifferentiation of adult epidermal cells to embryonic looking cells; (c) they induce a 200- to 400-fold increase in epidermal ornithine decarboxylase activity which specifically correlates with tumor promotion; (d) they induce protease activity; (e) they stimulate phospholipid synthesis and DNA, RNA, and protein synthesis; (f) they stimulate cellular proliferation; (g) they inhibit terminal differentiation not only in the skin but in other cellular systems such as Friend erythroleukemic cells, chicken myoblasts and neuroblastoma cells (Slaga *et al.*, 1978c). Only a limited number of studies have been performed concerning the mechanism by which other tumor promoters act. Both phenobarbitol and BHT stimulate DNA synthesis followed by cell proliferation in the liver and lung, respectively (Slaga *et al.*, 1978c).

Table 3. Cocarcinogens in various tissues.[a]

| | |
|---|---|
| **Skin** | |
| Catchol | benzo(*g*, *h*, *i*)perylene |
| pyrogallol | anthralin |
| lauryl alcohol | croton oil |
| decane | phorbol esters |
| undecane | phenols |
| tetradecane | nicotine |
| *n*-dodecane | surfactants |
| pyrene | radiation |
| benzo(*e*)pyrene,-B(*e*)P | viruses |
| fluoranthene | |
| **Lung** | |
| asbestos | ferric oxide |
| radiation | magnesium oxide |
| *n*-dodecane | hypoxia |
| **Mammary Gland** | |
| hormones | prolactin |
| estradiol | |
| **Bladder** | |
| L-tryptophan | cyclamate |
| saccharin | |
| **Cheek Pouch** | |
| X-radiation | croton oil |
| **Liver** | |
| cyclopropenoid fatty acids | alcohol |

[a]See Slaga *et al.* (1978c) for details.

## Materials and Methods

*Animals*

SENCAR mice, a mouse stock selected for its increased sensitivity to carcinogenesis as described by Boutwell (1964) were used in this study. These mice were derived from breeding Charles River CD-1 mice with male STS Skin Tumor Sensitive originally derived from Rockland mice by Boutwell (1964) and selecting for eight generations for sensitivity to DMBA-TPA two-stage system of tumorigenesis. These mice were initially obtained from Dr. Boutwell, McArdle Laboratory for Cancer Research, University of Wisconsin, Madison,

Wisconsin, and now are being raised at the Oak Ridge National Laboratory, Oak Ridge, Tennessee. In addition, C57 black mice obtained from Jackson Laboratory, Jackson, Maine are also being used for these studies. Since the sensitivity to skin cancer is much less than the SENCAR mice this report will only deal with the results from the SENCAR mice.

*Chemicals*

TPA was obtained from Dr. P. Borchert, University of Minnesota, Minneapolis, MN. DMBA was purchased from Sigma Chemical Co., St. Louis, MO; BP and pyrene from Aldrich Chemical Co., Milwaukee, WI. The various diesel emission particle extracts as well as the other environmental emission samples were obtained from EPA under a code designation. All the agents were consistently prepared under yellow light immediately before use. In addition, all samples were applied topically in 0.2 ml of spectral quality acetone.

*Tumor experiments*

The initial studies related to comparing tumor initiation and complete carcinogenesis of many carcinogens and the dose–response studies using DMBA, BP, and TPA involved the use of only SENCAR mice with 30 to 40 females per group. The other studies related to the various emission samples obtained from EPA utilized two strains of mice (SENCAR and C57 black) with 80 mice per treatment group (40 males and 40 females). In addition, 5 dose levels were used for the carcinogenic, tumor initiating, cocarcinogenic and tumor promoting activities of the various samples. BP was used as the standard for the complete carcinogenesis and tumor initiation studies and TPA for the tumor promotion studies. Pyrene was used as the positive control for cocarcinogenesis studies with BP as the carcinogen. See Table 4 for a summary of objective and protocol. All the mice were shaved with surgical clippers 2 days before the initial treatment and only those mice in the resting phase of the hair cycle were used. Skin tumor formation was recorded weekly and papillomas greater than 2 mm in diameter were included in the cumulative total if they persisted for 1 week or longer. Both the number of mice with tumors and the number of tumors per mouse were determined and recorded weekly. At random, papillomas and carcinomas were removed for histological verification.

Table 4. Objectives of the diesel research program.

| | |
|---|---|
| Determine: | Complete carcinogenesis |
| | Cocarcinogenesis |
| | Tumor initiation |
| | Tumor promotion |
| Samples: | Olds diesel |
| | Nissan diesel |
| | VW Rabbit diesel |
| | Caterpillar diesel |
| | Mustang gasoline catalyst |
| | Coke oven |
| | Roofing tar |
| | Cigarette smoke condensate |
| Protocol: | SENCAR and C57 black mice |
| | 40 males and 40 females |
| | 5 dose levels |
| Standards: | Benzo(*a*)pyrene for complete carcinogenesis and tumor initiation |
| | Pyrene for cocarcinogenesis with benzo(*a*)pyrene |
| | Phorbol ester (TPA) for tumor promotion |

## Results and Discussion

*Complete carcinogenesis vs tumor initiation*

Whenever a known skin carcinogen has been appropriately tested, it has shown skin tumor initiating activity (Boutwell, 1964; Slaga *et al.*, 1974a; Slaga *et al.*, 1974b; Slaga *et al.*, 1978a; Slaga *et al.*, 1978b; Slaga *et al.*, 1979; Slaga *et al.*, in press; Slaga *et al.*, 1976a; Slaga *et al.*, 1976b; Slaga *et al.*, 1973; Bowden *et al.*, 1974; Scribner and Slaga, 1975; Scribner, 1973; Hecht *et al.*, 1979; Cavalieri *et al.*, 1979). In a two-stage mouse skin system, initiation is the only stage that requires the presence of the carcinogen, and the measured carcinogenic potency of a chemical reflects its capacity for tumor initiation. The results in Table 5 show that there is both a good qualitative and quantitative correlation between the complete carcinogenic and tumor initiating activities of several chemical carcinogens in mouse skin. This is true when one considers the number of papillomas per mouse at early times (10 to 20 weeks) or the final carcinomas incidence after tumor initiation.

Table 5. Comparison of complete carcinogenesis and tumor initiation in mouse skin.[a]

| | Relative Potency[b] | |
|---|---|---|
| Compound | Complete Carcinogenesis (Carcinomas) | Tumor Initiation (Papillomas) |
| DMBA | 100 | 100 |
| MC | 50 | 50 |
| BP | 30 | 30 |
| 2-OHBP | 30 | 30 |
| 7BrMe-12MeBA | 20 | 20 |
| BP-7,8-oxide | 20 | 20 |
| DB(*a*,*h*)A | 20 | 20 |
| BA | 5 ± 5 | 5 |
| DB(*a*,*c*)A | 0 | 3 |
| Pyrene | 0 | 0 |
| BP-4,5-oxide | 0 | 0 |
| Anthracene | 0 | 0 |

[a] This is a summary of over 100 compounds which shows that an excellent qualitative and quantitative correlation exists between complete carcinogenesis and tumor initiation in mouse skin.
[b] Relative potency was determined from dose–response data. DMBA was given a maximum value of 100.

Table 6. Agents that are possibly pure tumor initiators.

| Skin Tumor Initiators | Relative Potency[a] |
|---|---|
| BP-7,8-diol-9,10-epoxide | 25 |
| MNNG | 15 |
| BA-3,4-diol-1,2-epoxide | 2 |
| BA | 0.5 |
| DB($a$, c)A | 0.2 |
| Chrysene | 0.1 |
| Urethane | 0.1 |

[a]Relative potency was determined from dose–response data. DMBA was given a maximum value of 100.

It is possible that a carcinogen lacking promoting ability would not be detected when tested as a complete carcinogen. In this regard, however, we have found a number of chemical compounds that have tumor initiating activity but lack complete carcinogenic activity (Slaga *et al.*, 1973; Scribner and Slaga, 1975; Slaga *et al.*, 1978b; Slaga *et al.*, 1979; Scribner, 1973). These pure skin tumor initiators are listed in Table 6. Due to these considerations, we feel that it is important to test a compound as a tumor initiator as well as a complete carcinogen. The major disadvantage of the skin system which is also true of other carcinogenesis systems is that many carcinogens are tissue specific.

There is a good dose–response relationship of many carcinogens used as tumor initiators in the two-stage carcinogenesis system using SENCAR mice. This is illustrated in Table 7. A good dose–response relationship exists for DMBA and B$a$P to initiate skin tumors in SENCAR mice. As can be seen a good correlation exists between the number of papillomas per mouse at 15 weeks and the final carcinoma incidence at 50 weeks. The percent of mice with papillomas has also a reasonable correlation but the dose response is very narrow. Table 8 shows the dose-response ability of TPA to promote tumors after DMBA initiation. As was the case for tumor initiation, there is also a very good dose-response relationship for tumor promotion when considering either the number of papillomas per mouse at 15 weeks or the percent of mice with squamous cell carcinomas at 50 weeks. Similar results have been reported using Charles River CD-1 mice (Slaga *et al.*, 1976a; 1974b) or ICR/Ha Swiss mice (Van Duuren *et al.*, 1973; 1978).

*Studies on the carcinogenesis of diesel emission particle extracts*

We were interested in determining the skin carcinogenic, cocarcinogenic, tumor initiating and promoting activities of diesel emission particle extracts. In addition, we wanted to compare the above results to other environmental emission particle extracts (coke oven, roofing tar, cigarette smoke and gasoline engine), as well as positive controls such as BP for tumor initiation and complete carcinogenesis, TPA for promotion and pyrene for cocarcinogenesis with BP. Presently, we can only present a preliminary comparative study on the skin tumor initiating activities of all the samples. Figure 1 shows that an excellent dose–response relationship exists for the initiating ability of BP at 14 weeks. A similar dose–response relationship exists for the tumor initiating activity of the Nissan diesel sample but using mg quantities for initiation (Fig. 2). The coke oven sample also gave a reasonable dose–response curve (Fig. 3). Table 9 summarizes the skin tumor initiating activities

Table 7. Dose–response studies on the ability of DMBA and BP to initiate skin tumors in SENCAR mice (the mice were treated one week after initiation with twice weekly applications of 5μg of TPA).

| Initiator | Dose (nmole) | # of papillomas per mouse at 15 weeks | % of mice with papillomas at 15 weeks | % of mice with carcinomas at 50 weeks |
|---|---|---|---|---|
| DMBA | 100 | 22 | 100 | 100 |
| DMBA | 10 | 6.8 | 100 | 40 |
| DMBA | 1 | 3.2 | 93 | 22 |
| DMBA | 0.1 | 0.5 | 20 | 5 |
| BP | 200 | 7.5 | 100 | 55 |
| BP | 100 | 3.2 | 78 | 30 |
| BP | 50 | 1.4 | 60 | 18 |

Table 8. Dose-response studies on the ability of TPA to promote tumors after DMBA initiation (the mice were initiated with 10 nmole of DMBA and promoted one week later with various dose levels of TPA).

| Promoter | dose (μg) | Time to first papilloma (wks) | # of papillomas per mouse at 15 weeks | % of mice with papillomas at 15 weeks | % of mice with carcinomas at 15 weeks |
|---|---|---|---|---|---|
| TPA | 10 | 8 | 3.0 | 100 | 32 |
| TPA | 5 | 6 | 7.2 | 100 | 46 |
| TPA | 2 | 7 | 6.5 | 100 | 45 |
| TPA | 1 | 8 | 3.6 | 80 | 25 |
| TPA | 0.1 | 11 | 0.4 | 5 | 8 |

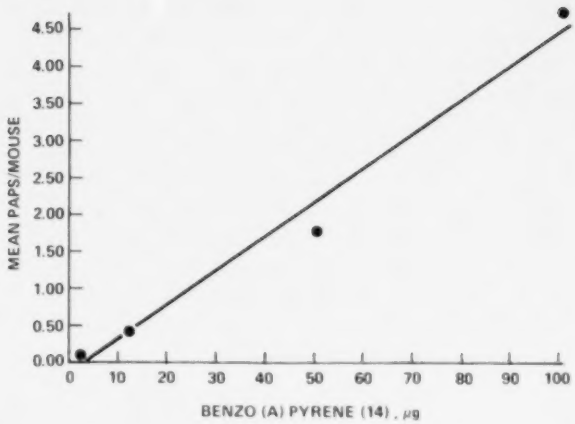


Fig. 1. Dose–response relationship for BP applied topically to male and female SENCAR mice once and followed 1 week later by twice weekly application of 2 μg of TPA for 14 weeks.

of the various samples. The comparison of their tumor initiating activities was made at 14 weeks. The tumor response is expressed as the mean number of papillomas per mouse per mg. The values represent the slope from a linear regression analysis when plotting data from dose–response studies. Caterpillar diesel and cigarette smoke condensate were for all practical purposes zero whereas Mustang gasoline and Olds diesel gave extremely low values, although they were above background. Roofing tar, Nissan diesel, and coke oven all gave moderate activity at high doses (1 to 10 mg) but when compared to benzo(*a*)pyrene were relatively low values. Ten mg of the roofing tar sample had a tumor initiating activity comparable to 50 μg of BP, whereas 10 mg of the Nissan diesel was comparable to 60 μg of BP, and 10 mg of the coke oven sample was comparable to 80 μg of BP.

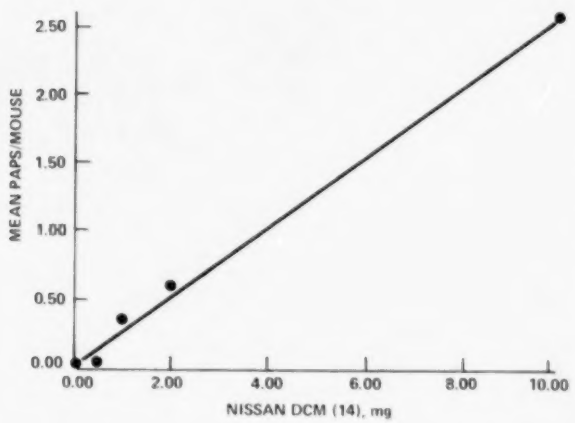


Fig. 2. Dose–response relationship for the Nissan diesel sample (Nissan DCM) applied topically to male and female SENCAR mice and followed 1 week later by twice weekly applications of 2 μg of TPA for 14 weeks.

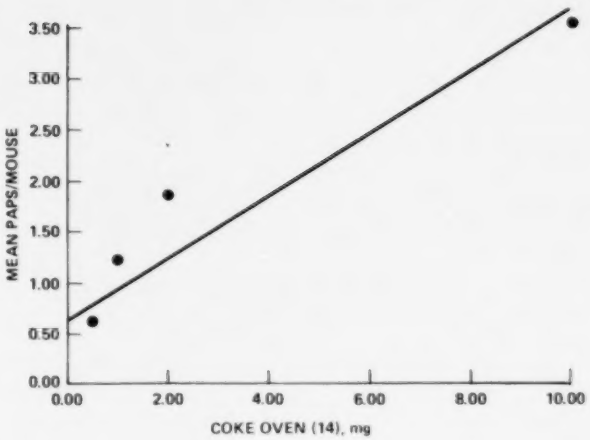


Fig. 3. Dose–response relationship for the coke oven sample applied topically to male and female SENCAR mice and followed 1 week later by twice weekly applications of 2 μg of TPA for 14 weeks.

## Abbreviations

TPA, 12-0-tetradecanoylphorbol-13-acetate; DMBA, 7,12-dimethyl-benz(*a*)anthracene; BP, benzo(*a*)pyrene; MC, 3-methylcholanthrene; 2-OHBP, 2-hydroxybenzo-(*a*)pyrene; 7BrMe-12MeBa, 7-bromomethyl-12-methyl-benz(*a*)anthracene; BP-7,8-oxide, benzo(*a*)pyrene-7,8-oxide; DB(*a*, *h*)A, dibenz(*a*, *h*)anthracene; BA, benz-(*a*)anthracene; DB(*a*, *c*)A, dibenz(*a*, *c*)anthracene; BP-4,5-oxide, benzo(*a*)pyrene 4,5-oxide; BP-7,8-diol-9,10-epoxide,(±)-7β,8α-dihydroxy-9α-10α-epoxy-7,8,-9,10-tetrahydrobenzo(*a*)pyrene; MNNG, N-methyl-N′-nitro-N-nitrosoguanidine; BA-3,4-diol-1,2-epoxide; (±)-3α,4β-dihydroxy-1α,2α-epoxy-1,2,3,4-tetrahydro-benz(*a*)anthracene.

*Acknowledgements*—Research sponsored by U.S. EPA contract Number 79-D-X0526 under Interagency Agreement DOE Number 40-728-78 and the Office of Health and Environmental Research, U.S. Department of Energy, under contract W-7405-eng-26 with the Union Carbide Corporation.

Table 9. Comparative skin tumor initiating activities of extracts of diesel and related environmental emissions.[a]

| Sample | ± Papillomas/Mouse/mg[b] (14 weeks) | $R^2$ |
|---|---|---|
| Caterpillar diesel | 0 | |
| Nissan diesel | 0.258 | 0.996 |
| Olds diesel | 0.115 | 0.95 |
| Mustang gasoline | 0.09 | — |
| Cigarette smoke condensate | 0 | — |
| Roofing tar | 0.182 | 0.999 |
| Coke oven emissions | 0.307 | 0.876 |
| Benzo(*a*)pyrene | 46.2 | 0.984 |

[a]40 males and 40 females were initiated with the various samples and promoted one week later by twice weekly applications of 2 μg of TPA.
[b]The values represent the slope from the linear regression analysis of the dose–response studies and measure of fit ($R^2$).

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